K-252a, A POTENT INHIBITOR OF PROTEIN KINASE C FROM MICROBIAL ORIGIN

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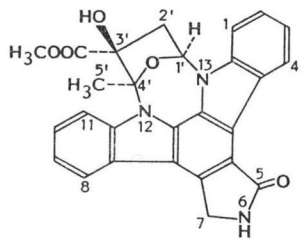
K-252a, a metabolite isolated from the culture broth of *Nocardiopsis* sp. K-252a, was found to exhibit an extremely potent inhibitory activity on protein kinase C. The IC_{50} value was 32.9 nM.

The functional role of Ca^{2+} is well recognized as a second messenger for control of a variety of cell functions such as secretion, contraction, phototransduction, cell division and differentiation, and alteration of the transport of ions[1]. Many of the effects of Ca^{2+} are transmitted by calmodulin, or other members of the same family of Ca^{2+} binding protein[2~4]. In addition, the recent discovery of a phospholipid-sensitive Ca^{2+}-dependent protein kinase (protein kinase C) by NISHIZUKA and co-workers[5] has led to a deeper understanding of how the Ca^{2+}-messenger system operates. There appear to be two branches by which various extracellular informational signals flow from the cell surface to the cell interior[6,7]. One is mediated by a rise in the $[Ca^{2+}]$ concentration in the cell cytosol, leading to the modulation of the function of calmodulin-dependent reactions, the other by a rise in the diacylglycerol content of the plasma membrane, leading to the activation of protein kinase C. Both routes usually become available as the result of an interaction of a signal ligand and a receptor, and act synergistically to evoke subsequent cellular responses[6]. Furthermore, the two branches may have unique roles in the temporal integration of cellular response in several cell types[7].

In the course of studies on inhibitors or antagonists of the Ca^{2+}-messenger system, we found that a novel metabolite of *Nocardiopsis* sp. K-252 inhibited protein kinase C and calmodulin. The compound, designated K-252a (previously named K-252,) was isolated from the culture broth, and its structure was determined to be **1**[8],† (Fig. 1). K-252a exhibited an extremely potent inhibitory activity of protein kinase C. The compound also inhibited calmodulin-activated enzymes, although its effect on calmodulin was much less than that on protein kinase C.

Fig. 1. The structure of K-252a (**1**).



In this communication, we describe the fermentation, isolation and purification, and some biochemical properties of K-252a. Structural elucidation studies will be reported in a subsequent paper[10].

† Recently, SEZAKI *et al.*[9] reported to isolate a compound SF-2370 from the culture broth of *Actinomadura* sp; the structure of SF-2370 was identical to K-252a.

Materials and Methods

Materials

Phosphatidylserine was purchased from Serdary Research Laboratories; diolein was from Nakarai Chemicals Ltd., Japan; [γ-^{32}P]ATP was from Amersham Corp.; histone H1 (type III-S, from calf thymus), cAMP, calmodulin and its dependent cyclic nucleotide phosphodiesterase (PDE) from bovine heart, and 5'-nucleotidase (*Crotalus atrox* venom) were from Sigma Chemical Co.

Microorganisms

Nocardiopsis sp. K-252 (NRRL 15532), isolated from a soil of Ashahi-cho, Machida-shi, Tokyo, Japan, was employed in the present investigation. Morphological, cultural and physiological characteristics of the strain will be described in detail elsewhere.

Fermentation

A culture tube containing 15 ml of a seed medium composed of glucose 0.5%, soluble starch 3.0%, soybean meal 2.0%, yeast extract 0.5%, corn steep liquor 0.5%, $CaCO_3$ 0.3% (pH 7.2 before sterilization) was inoculated with the mycelia of the organism grown on agar slant. The composition of the agar slant medium (Hickey-Tresner) was as follows; soluble starch 1%, N-Z amine type A 0.2%, beef extract 0.1%, yeast extract 0.1%, and agar 2% (pH 7.2). The inoculated tube was incubated on a reciprocating shaker (300 rpm) at 28°C for 96 hours. A 4-ml-aliquot of the culture was inoculated into a 300-ml Erlenmeyer flask containing 40 ml of the same medium and incubated on a rotary shaker (200 rpm) at 28°C for 72 hours. A 30-ml-aliquot of the culture was transferred into a 2-liter Erlenmeyer flask containing 300 ml of the same medium and incubated at 28°C for 120 hours. The microbial growth was used to inoculate a 30-liter jar fermentor containing 18 liters of the same medium. Fermentation was carried out for 160 hours with agitation at 200 rpm and aeration of 18 liter/minute. K-252 production in the culture filtrate was quantified spectrophotometrically after developing the filtrate on TLC plate (Merck, Kieselgel 60 F_{254}, 5715) with $CHCl_3$ - MeOH (9 : 1). The plate was scanned at the wavelength of 292 nm with a Shimadzu Dual-wavelength TLC Scanner.

Preparation of Protein Kinase C

Protein kinase C was partially purified from rat brain cytosol by a modification of the procedures described by KIKKAWA *et al.*[11]. In brief, cerebral cortices of male Sprague-Dawley rats, weighing 150 to 200 g, were homogenized in 20 mM Tris-HCl buffer (pH 7.5) containing sucrose 250 mM, EDTA 2 mM, EGTA 10 mM and phenylmethyl sulfonyl fluoride 2 mM (Buffer A). The homogenate was centrifuged at 100,000 $\times g$ for 60 minutes at -4°C and the supernatant was applied to a DE52 column (Whatman, 100 ml). The column was washed with 300 ml of 20 mM Tris-HCl buffer (pH 7.5) containing EDTA 2 mM, EGTA 5 mM, and 2-mercaptoethanol 50 mM (Buffer B) and then with the same buffer containing EDTA 1 mM, EGTA 1 mM and 2-mercaptoethanol 50 mM (Buffer C). The enzyme was eluted by application of a 1,200 ml linear gradient of NaCl (0 to 0.3 M) in Buffer C at a flow rate 75 ml/hour. The fractions containing the enzyme were pooled and concentrated in an Amicon ultrafiltration cell equipped with PM-10 filter membrane. The concentrated enzyme was stored at -80°C in the presence of 50% (w/v) glycerol. By these procedures, protein kinase C was purified about 8.2-fold from the crude extract with an overall recovery of about 14%. The enzyme preparation contained no detectable cAMP- or cGMP-dependent protein kinase activity.

Preparation of Calmodulin and Its Dependent PDE from Bovine Brain

Calmodulin and its dependent PDE were partially purified from bovine brain by a modification of the procedures described by KAKIUCHI *et al.*[12]. Cerebral cortices of bovine brain were homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing $MgSO_4$ 1 mM, EGTA 0.1 mM and 2-mercaptoethanol 5 mM. The homogenate was centrifuged at 24,000 $\times g$ for 60 minutes at 0°C and the supernatant was centrifuged again at 113,000 $\times g$ for 60 minutes. The final supernatant was applied to a DEAE-Sephacel column (Pharmacia, 5 $\times$ 30 cm). The column was washed with 10 mM Tris-HCl buffer (pH 6.0) containing $MgSO_4$ 1 mM, EGTA 0.2 mM and 2-mercaptoethanol 5 mM, and then eluted with a

linear gradient of CH_3COONa (0 to 1 M) in the same buffer. The fractions containing calmodulin or its dependent PDE were pooled and concentrated by ultrafiltration through PM-10 membrane. The concentrated enzyme and calmodulin were dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.5) containing $MgSO_4$ 1 mM, EGTA 0.1 mM, 2-mercaptoethanol 5 mM and NaCl 100 mM, and centrifuged at 27,000 × *g* for 30 minutes. The supernatant was stored at −80°C in small aliquots.

Enzyme Assay and Determination

Protein kinase C was assayed essentially as described by KIKKAWA *et al.*[11]. Briefly, the incubation mixture (0.25 ml) contained Tris-HCl buffer (pH 7.5) 5 μmol, magnesium acetate 2.5 μmol, histone H1 50 μg, [γ-^{32}P]ATP 1.25 nmol, phosphatidylserine 20 μg, 1,2-diolein 0.8 μg, $CaCl_2$ 0.75 μmol, EGTA 1 μmol, and the enzyme. After incubation at 30°C for 3 minutes, the reaction was stopped by the addition of 3 ml of 25% trichloroacetic acid. The acid precipitates were collected on a Toyo-Roshi membrane filter, and the radioactivity of the filter was measured using liquid scintillation counter. To determine the effect of a drug on the enzyme activity, 5 μl of the drug solution was added to the reaction mixture.

PDE activity was determined as described by KAKIUCHI *et al.*[12] based on the method of BUTCHER and SUTHERLAND[13]. The reaction mixture contained, in a final volume of 0.5 ml, imidazole-HCl buffer (pH 6.9) 80 mM, $MgSO_4$ 3 mM, dithiothreitol 0.3 mM, NaCl 100 mM, cAMP 1.2 mM as a substrate, the enzyme (PDE) and other additions as indicated. The reaction was started by adding the enzyme. Incubation was carried out at 30°C for 30 minutes, and the reaction was stopped by boiling for 5 minutes. Then 6 μmol of $MnCl_2$ and a sufficient amount of 5′-nucleotidase was added and the mixture was incubated at 30°C for another 30 minutes. The reaction was terminated by adding 3 ml of 10% perchloric acid, and the mixture was centrifuged at 1,300 × *g* for 5 minutes. Inorganic phosphate liberated in the supernatant was measured by the method of AMES[14].

In the determination of the Ca^{2+}/calmodulin-activated activity (total activity), $CaCl_2$ (50 μM) and calmodulin (as indicated in legends of Figs.) were added to the assay mixture. Basal activity was similarly determined, but in the presence of 3 mM EGTA instead of Ca^{2+} and calmodulin. The Ca^{2+}/calmodulin-activated activity was expressed in terms of total activity minus basal activity. In this investigation, a unit of PDE was defined as the amount which hydrolyses 1 μmol of cAMP per minute at 30°C and a unit of calmodulin as the amount which gives 50% stimulation of PDE activity. To determine the effect of a drug on the enzyme activity, the drug solution (50 μl) was added to the reaction mixture prior to the addition of the enzyme.

To determine the inhibitory effect of a drug on basal activity, a large amount of PDE (9.3 mU; unit definition was determined in the absence of calmodulin) was used to magnify PDE activity. The actual effect induced by the drug was corrected by subtracting the value obtained for the vehicle.

Protein was determined according to BRADFORD[15].

Results

Fermentation, Isolation and Purification, and Physico-chemical Properties

Time course of K-252a production by *Nocardiopsis* sp. K-252 in a 30-liter jar fermentor is shown in Fig. 2. The amount of K-252a in the culture supernatant increased gradually from 0 to 120 hours of cultivation and then rapidly increased with the concomitant lysis of cells.

The procedure for isolation of K-252a is shown schematically in Fig. 3. Culture broth (15 liters) was centrifuged in a Sharples centrifuge. The supernatant was applied onto a Diaion HP-10 resin column (2 liters) and washed with 50% methanol (4 liters), and then with 30% acetone (2 liters). Adsorbed material was eluted with acetone (4 liters). The eluate was concentrated *in vacuo* and extracted with ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and then concentrated *in vacuo* to yield oily yellow material (4.9 g). The oily material was applied to silica gel column chromatography. Elution was performed with chloroform, and then with chloroform - methanol (98: 2). K-252a was

Fig. 2. Time course of K-252a production in a 30-liter jar fermentor.

○; K-252a, ●; OD_{660} (optical density at 660 nm, the value of culture broth diluted 100-fold), ▲; pH.

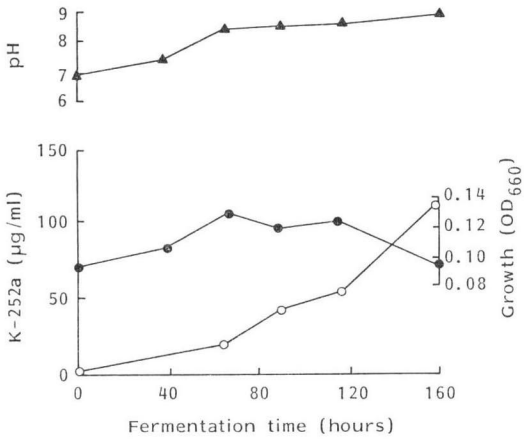


Table 1. Physico-chemical properties of K-252a.

Appearance	Pale yellow crystals
TLC* (Rf)	
$CHCl_3$	0.5
EtOAc	0.35~0.55
$CHCl_3$ - MeOH (9:1)	0.6~0.7
MeOH	0.7~0.8
Color reaction	
Positive	Anisaldehyde
Negative	$FeCl_3$, Ninhydrin, Rydon-Smith
Solubility	
Soluble	$CHCl_3$, pyridine, THF, DMSO, CH_3CN, Me_2CO
Slightly soluble	MeOH, EtOH, EtOAc, BuOH, PrOH
Insoluble	H_2O

* Silica gel (Merck, 5715).

Fig. 3. Purification procedure of K-252a.

Culture broth (15 liters)
- centrifuged

Supernatant
- Diaion HP-10 column chromatography (2 liters)
  - washed with 50% MeOH (4 liters) and then 30% acetone (2 liters)
  - eluted with acetone (4 liters)
  - concd
  - extracted with EtOAc

EtOAc layer
- dried over Na_2SO_4
- concd

Silica gel column chromatography (Wakogel C-200, 150 ml)
- washed with $CHCl_3$ (800 ml)
- eluted with $CHCl_3$ - MeOH (98: 2, 700 ml)

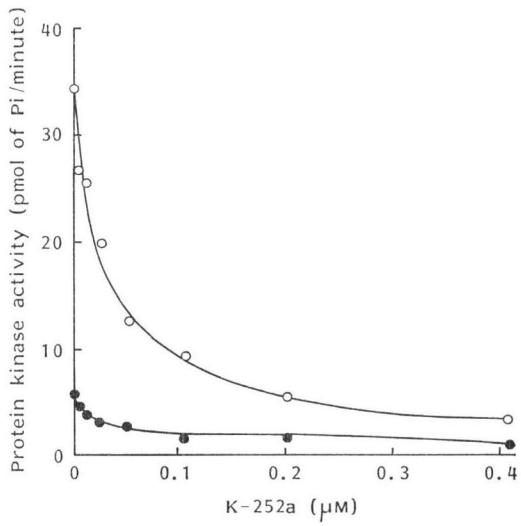
K-252a fractions
- concd
- dissolved in $CHCl_3$
- added MeOH

K-252a (pale yellow crystals, 610 mg)

Fig. 4. Inhibition of protein kinase C by K-252a.

Assay conditions were as described under "Materials and Methods", with various concentrations of K-252a added as indicated.

Protein kinase C (40 μg) was incubated (0.25 ml) in the presence of EGTA (1 μmol) with (○) or without (●), $CaCl_2$ (0.75 μmol), phosphatidylserine (20 μg) and diolein (0.8 μg).



eluted with the latter. Fractions containing K-252a was collected and concentrated *in vacuo* to dryness. The yellow powder thus obtained was dissolved in chloroform and methanol was added. The solution was kept standing at 4°C to crystallize K-252a (610 mg).

K-252a was obtained as pale yellow crystals, melting at 262~273°C. It is readily soluble in acetone, pyridine, chloroform, tetrahydrofuran, acetonitril and dimethyl sulfoxide, sparingly soluble in

Table 2. Effect of K-252a on cyclic nucleotide phosphodiesterases.

Enzymes	CaM*	IC_{50} (μM)
Bovine brain CaM-PDE**	+[a]	2.9
	−[b]	>200
Bovine heart CaM-PDE**	+[c]	1.3
Bovine heart CaM-independent PDE	−[d]	97.5

[a] The activity in the presence of 4 U/ml calmodulin, and 50 μM $CaCl_2$. The enzyme concentration was 26 mU/ml.

[b] The activity in the presence of 3 mM EGTA, without $CaCl_2$ and calmodulin. The enzyme concentration was 18.6 mU/ml.

[c] The activity in the presence of 2.5 U/ml calmodulin, and 50 μM $CaCl_2$. The enzyme concentration was 40 mU/ml.

[d] The activity in the presence of 3 mM EGTA, without $CaCl_2$ and calmodulin. The enzyme concentration was 25 mU/ml.

* Calmodulin. ** Ca^{2+}/calmodulin-dependent phosphodiesterase.

alcohol and ethyl acetate, and virtually insoluble in water. The Rf values of the compound on silica gel TLC developed in various solvent systems are shown in Table 1. The structure of K-252a was determined to be **1** (Fig. 1) on the basis of physico-chemical analyses, and various spectral and X-ray crystallographic data[10].

Biochemical Properties

Fig. 4 shows the effect of various concentrations of K-252a on protein kinase C from rat brain. K-252a inhibited the Ca^{2+}/phospholipid-dependent activity of the enzyme in a concentration-related manner. The IC_{50} value, (the concentration causing 50% inhibition), under present assay conditions, was 32.9 nM. Basal activity of the enzyme (the activity in the absence of Ca^{2+}, phosphatidylserine and diolein) was inhibited similarly by K-252a.

K-252a inhibited Ca^{2+}/calmodulin-stimulated PDE's from bovine brain and heart (Table 2). The activity of K-252a for calmodulin was much less than that for protein kinase C. The IC_{50} values for the effect of the enzymes were; brain PDE, 2.9 μM and heart PDE, 1.3 μM. K-252a inhibited specifically the Ca^{2+}/calmodulin-dependent activities of the enzymes without appreciably affecting their basal activities. In addition, calmodulin-independent PDE from bovine heart was only weakly inhibited by K-252a (IC_{50} value, 97.5 μM).

K-252a at 100 μg/ml exhibited no antimicrobial activity against *Candida albicans* KY5011, *Enterococcus faecalis* KY4280, *Pseudomonas aeruginosa* KY4276, *Staphylococcus aureus* KY4779, *Escherichia coli* KY4271, *Bacillus subtilis* KY4773, *Proteus vulgaris* KY4277, *Shigella sonnei* KY4281, *Salmonella typhosa* KY4278, or *Klebsiella pneumoniae* KY4275.

No acute toxicity of the compound was observed at 100 mg/kg in mice injected intraperitoneally.

Discussion

Several kinds of compounds have been reported to inhibit protein kinase C. A variety of lipid-interacting agents including chlorpromazine, imipramine, phentolamine, dibucaine, verapamil and tetracaine are able to inhibit protein kinase C[16] to various degrees. Phenothiazines[17,18], such as trifluoperazine and chlorpromazine, *N*-(6-aminohexyl)-5-chloronaphthalenesulfonamide (W-7)[19~21], and R-24571 (calmidazolium)[22], known as calmodulin inhibitors, have also been shown recently to inhibit protein kinase C. From these observations, SCHATZMAN *et al.*[23] suggested that there appeared

to be little selectivity for inhibition of Ca^{2+}/calmodulin- or Ca^{2+}/phospholipid-dependent enzyme by these agents. More recently, HIDAKA *et al.*[24] reported that isoquinolinesulfonamides, such as H-7, inhibit protein kinase C and cyclic nucleotide-dependent protein kinases, competing with ATP. Now, we have found that K-252a, a novel metabolite isolated from *Nocardiopsis* sp. K-252, inhibits protein kinase C. K-252a appears to be the most potent inhibitor of protein kinase C ever reported; the IC_{50} value for K-252a was 32.9 nM, which is two or three orders of magnitude lower than that for the protein kinase C inhibitors described above (the IC_{50} values for H-7, trifluoperazine, chlorpromazine, and *R-24571* were 12, 16, 24 and 5.3 μM, respectively, under comparable incubation conditions). Furthermore, the inhibitory effects by K-252a seem to be attributed to the direct interaction of the compound with the enzyme, and not with the enzyme activators, as suggested by its inhibition of the basal activity of the enzyme. Detailed kinetic analysis will be described in a separate paper. Whether K-252a inhibits other protein kinases such as cAMP- and cGMP- dependent protein kinases, remains to be determined. The inhibitory specificity of K-252a for protein kinases is under investigation.

K-252a also inhibits calmodulin. Although the inhibitory activity for calmodulin is much less than that for protein kinase C, it may be of interest to determine whether or how these calmodulin-antagonistic properties contribute to the *in vitro* or *in vivo* pharmacological effect of K-252a on Ca^{2+}-messenger systems in various cells and tissues. We have found that K-252a seriously affects the function of various cells and tissues, such as platelets[25], mast cells[9] and vascular smooth muscle. This newly developed compound may be a useful tool for clarifying the *in vitro* and *in vivo* functions of protein kinases and/or calmodulin.

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